



## Medical and Veterinary Entomology

# Effect of *Bacillus sphaericus* Neide on *Anopheles* (Diptera: Culicidae) and associated insect fauna in fish ponds in the Amazon



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## ABSTRACT

We analyzed the effects of *Bacillus sphaericus* on *Anopheles* larvae and on the associated insect fauna in fish farming ponds. Five breeding sites in the peri-urban area of the city of Manaus, AM, Brazil, were studied. Seven samples were collected from each breeding site and *B. sphaericus* was applied and reapplied after 15 days. The samples were made at 24 h before application, 24 h post-application and 5 and 15 days post-application. We determined abundance, larval reduction and larval density for *Anopheles*, and abundance, richness, Shannon diversity index and classified according to the functional trophic groups for associated insect fauna. A total of 904 *Anopheles* larvae were collected and distributed into five species. Density data and larval reduction demonstrated the rapid effect of the biolarvicide 24 h after application. A total of 4874 associated aquatic insects belonging to six orders and 23 families were collected. Regression analysis of diversity and richness indicated that the application of the biolarvicide had no influence on these indices and thus no effect on the associated insect fauna for a period of 30 days. *B. sphaericus* was found to be highly effective against the larvae of *Anopheles*, eliminating the larvae in the first days after application, with no effect on the associated insect fauna present in the fish ponds analyzed.

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## Introduction

The Amazon environment is rich in water resources, very common mostly due to the existing mesh of rivers, thus enabling the formation of numerous breeding sites for many groups of aquatic organisms (Sioli, 1984). These organisms particularly include insects, where some of them are vectors of pathogens that cause human tropical diseases. Accordingly, mosquitoes occupy a central role because of their plasticity in colonizing different aquatic environments, high density in this environment and food preference for human blood (Tadei and Thatcher, 2000; Forattini, 2002).

Control of the disease, according to the National Program for Prevention and Control of Malaria Brazil, has among other measures, early diagnosis and treatment of patients, major steps for lifting the movement of parasite. They are also recommended vector control measures: indoor residual insecticide application, the treatment of breeding sites with biolarvicides, the use of impregnated mosquito

nets and long term, in special situations, spatial fogging of insecticides (Ministério da Saúde/SVS, 2003; Oliveira-Ferreira et al., 2010).

Breeding sites play an essential role in the maintenance of the disease, since adults emerge ready for their daily blood meal (Rodrigues et al., 2008). The control of immature forms can be performed using chemical larvicides, but this technique is not used due to the risk of development of resistance and environmental contamination, and therefore, the use of biological larvicides is increasing. The main representatives of these larvicides are toxic crystal-producing bacteria of the genus *Bacillus* (Galardo et al., 2013). The use of the species *Bacillus sphaericus* Neide, 1904 for mosquito control was advocated in 1985 by the World Health Organization. Since then, larvicides have been produced with this bacterium due to its recognized toxicity to the genera *Anopheles* and *Culex* (Habib, 1989; De Barjac, 1990).

The application of biological larvicides to control immature *Anopheles* sp. is carried out directly at the breeding sites (De Barjac, 1990). However, these environments also have an associated insect fauna, which is formed by several other groups of aquatic insects that share the same habitat as these mosquitoes (Lang and Reymond, 1994). About 13 orders of insects have an aquatic phase, and in some biotopes, they may comprise around

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95% of the macroinvertebrate community. These invertebrates play a key role in the health of the water body, by participating in the cycling of nutrients and the transformation of organic matter, contributing to the flow of energy (Brasil et al., 2014).

The effect of *B. sphaericus* on the associated insect fauna under laboratory and field conditions has been investigated over the past decades, and most of these organisms have not shown any susceptibility to the bacteria (Mulla et al., 1984; Aly and Mulla, 1987; Karch et al., 1991; Rodcharoen et al., 1991; Brown et al., 2004; Merritt et al., 2005).

Rodrigues et al. (2008) conducted field tests in Manaus, Brazil, applying *B. sphaericus* in fish ponds and standing water in pottery, and observed the elimination of larvae at breeding sites 48 h after application. Rodrigues et al. (2013) investigated the effects of *B. sphaericus* in applications on the Negro and Solimões Rivers, where it was found more effective in the black water river than the white water, which has a higher amount of suspended material.

Studies of the effect of *B. sphaericus* against *Anopheles* larvae have been conducted in Brazil, but there is a gap in our knowledge of its action on the associated insect fauna, particularly in the Amazon environment. This study aimed to analyze the effects of *B. sphaericus* on *Anopheles* and the associated insect fauna in fish farming ponds in the peri-urban area of the city of Manaus, Amazonas.

## Material and methods

### Bioassays in field

The sampling sites are located in the peri-urban region of Manaus, Central Amazonia in northern Brazil. Five artificial breeding sites of *Anopheles*, namely fish culture ponds, were selected: C1 (S 03°04'32.2", W 59°53'07.4"); C2 (S 03°02'07.6", W 59°53'30.2"); C3 (S 03°03'45.8", W 59°51'11.3"); C4 (S 03°02'44.4", W 59°53'09.0") and C5 (S 03°02'43.9", W 59°53'09.0") (Fig. 1). The larvicide used was VECTOLEX CG®, formulated granules (Valent BioSciences Corporation), concentrated dried serotype H5a5b at 7.5% with a power of approximately 670 Bs international toxin units (ITU), containing corn oil and corn cob granules. We used the dose recommended by the manufacturer, i.e., 11 kg/ha.

The applications were done manually by reaching across the edge and over a perimeter of 3 m of the breeding center. Field bioassays lasted 30 days at each pond and two applications (application and reapplication) of biolarvicide were performed with an interval of 15 days between them. Samples of *Anopheles* larvae and associated insect fauna were obtained at the following times: pre-application – sampling performed before application; and after application of biolarvicide – three post-application samples and three post-reapplication samples at the following times: 24 h, 5 days and 15 days. The period of larvicide application began in December 2011 and lasted until April 2012, where there were 9 field trips at each breeding site, totaling 45 for the whole bioassay.

### Sampled of insects

*Anopheles* larvae were collected for 20 min at three randomly selected points on the edge of each breeding site using an entomological scoop with volumetric capacity of about 350 mL, 11 cm aperture and capable of handling a meter. During sampling, the 4th instar larvae of *Anopheles* were separated and taken to the Malaria and Dengue Laboratory of the National Institute of Amazonian Research, maintained under laboratory conditions and grown to adulthood to facilitate species identification. The identification of *Anopheles* was performed using the dichotomous key proposed by Consoli and Oliveira (1994).

Aquatic insects were collected using an aquatic insect net, at four random points for 30 s at each point (Merritt et al., 2005). Subsequently, the material was fixed in 70% alcohol and brought to the laboratory where it was screened with the aid of a stereomicroscope. The insects found were identified to the lowest possible taxonomic level using dichotomous keys (Merritt and Cummins, 1996; Pes et al., 2005; Pereira et al., 2007). Subsequently, the individuals were classified according to the functional trophic groups separated into shredders, scrapers, collectors, filter feeders and predators, following recommendations by Merritt and Cummins (1996) and Cummins et al. (2005).

### Data analysis

To characterize the insect fauna, the relative abundance (%) of aquatic insects and species of *Anopheles* (Magurran, 1988) was calculated. To evaluate the effect of *B. sphaericus* on *Anopheles*, larval reduction (%) was obtained by using the three post-application and post-reapplication data. The index uses the number of larvae before and after application of biolarvicide, obtaining the percent reduction of *Anopheles* larvae during the experiment (Mulla et al., 1986). Larval density (LNMH) was determined before and after application of biolarvicide at breeding sites LNMH was obtained according to the formula described by Tadei et al. (2007) and was superimposed on abundance data over time insect fauna associated to check the behavior of these populations during the biolarvicide action period.

To evaluate the effect of *B. sphaericus* on insect fauna associated vectors, richness and diversity data were analyzed by linear regression analysis, with the aid of the Statistica Statsoft 10.0 program. The independent variable was the application of *B. sphaericus* and dependent was the richness and diversity of associated insect fauna. The relationship between richness/diversity and the use of biolarvicide was assessed by regression models that best fit the data distribution. The values obtained after two biolarvicide application cycles at the five artificial breeding sites were taken into account.

## Results

A total of 905 larvae of *Anopheles* were collected identified in five species: *Anopheles darlingi*, *Anopheles albitarsis*, *Anopheles braziliensis*, *Anopheles triannulatus* and *Anopheles nuneztovari*. The relative abundance showed that *A. darlingi* (54%) was the most abundant species and *A. albitarsis* (1%) the least. Considering the associated insect fauna, 4874 specimens belonging to 6 orders and 23 families were collected. Chironomidae was the most abundant family with 51%, followed by Ceratopogonidae (14%) and Coenagrionidae (11%), and the least abundant was Gerridae (0.02%) (Table 1). Among the functional trophic groups, the collectors were the most abundant in three of the five breeding sites analyzed (C2: 85%, C3: 45.5% and C4: 60%); in the other two, the predators were the most abundant (C1: 73.5% and C5: 68%). The group of shredders was the least abundant (C4: 0.2%) in all breeding sites analyzed.

### Effects on *Anopheles*

At three of the five treated breeding sites (C1, C2 and C5), the larvae were eliminated at 24 h (100%) and up to 5 days after the application of biolarvicide, and at C4, the reduction was 98%. After reapplication, the larval reduction rate was high in the initial readings but decreased 15 days after re-application. At breeding site C3, 24 h after *B. sphaericus* application, larval reduction was 56%, and at five days, negative values were obtained for larval reduction, because there was an increase in larvae. After reapplication, 100% reduction in larvae was observed after 24 h and also after 15 days (Table 2).

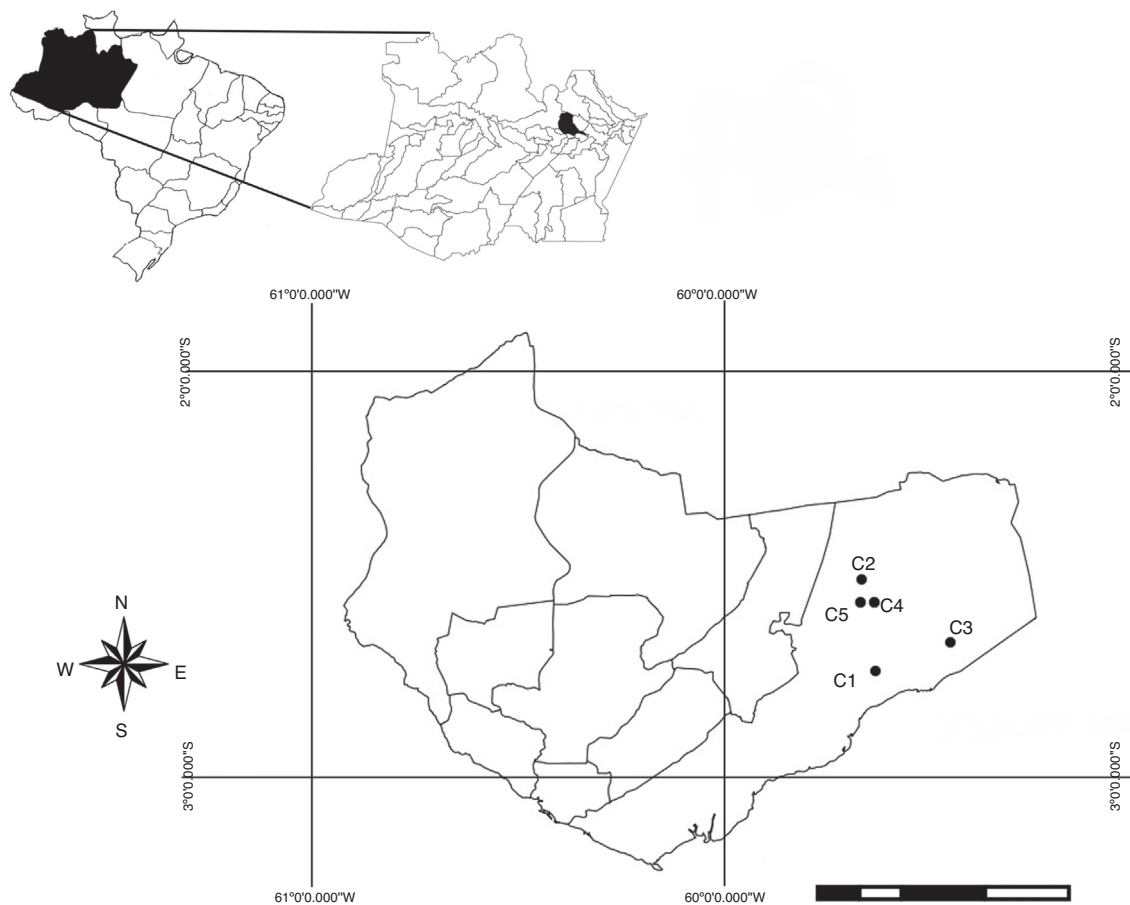


Fig. 1. Location of breeding sites for applications of *B. sphaericus*, Manaus, Amazonas, Brazil.

**Table 1**  
Relative abundance (%) of aquatic insect fauna associated with anophelines found in fish ponds during applications of *Bacillus sphaericus*, Manaus, Amazonas.

Order	Family	Fish ponds					n	R.A.%
		C1	C2	C3	C4	C5		
Diptera	Ceratopogonidae	8	186	43	211	238	686	14
	Chironomidae	325	1341	143	528	169	2506	51
	Culicidae	8	11	12	1	2	34	0.6
Odonata	Libellulidae	294	13	31	9	3	350	7.1
	Coenagrionidae	520	6	22	22	13	583	11
	Corduliidae	–	2	2	2	1	7	0.1
Hemiptera	Belostomatidae	84	–	21	45	97	247	5
	Notonectidae	23	14	4	–	7	48	0.9
	Veliidae	16	1	1	2	5	25	0.5
	Pleidae	8	–	–	–	–	8	0.1
	Nepidae	8	–	–	4	5	17	0.3
	Corixidae	4	–	33	23	6	66	1.3
	Micronectidae	–	–	1	1	5	7	0.14
Coleoptera	Gerridae	–	–	–	–	1	1	0.02
	Noteridae	–	2	1	30	5	38	0.7
	Hydrophilidae	47	2	–	5	17	71	1.4
	Elmidae	9	1	–	1	–	11	0.2
	Dytiscidae	25	7	6	3	3	44	0.9
Ephemeroptera	Caenidae	33	11	6	5	3	58	1.1
	Baetidae	–	29	4	13	1	47	0.9
	Polymitarcyidae	–	1	–	4	7	12	0.2
Trichoptera	Hydroptilidae	–	–	6	–	–	6	0.1
	Leptoceridae	–	–	–	2	–	2	0.04
	Total						4874	

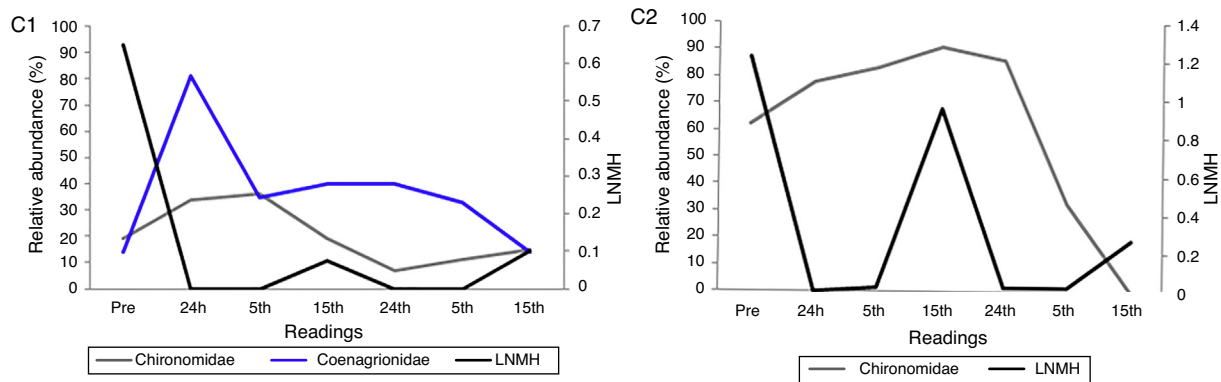


Fig. 2. Abundance over time of aquatic insects and values LNMH at C1 and C2, Manaus, Amazonas, Brazil.

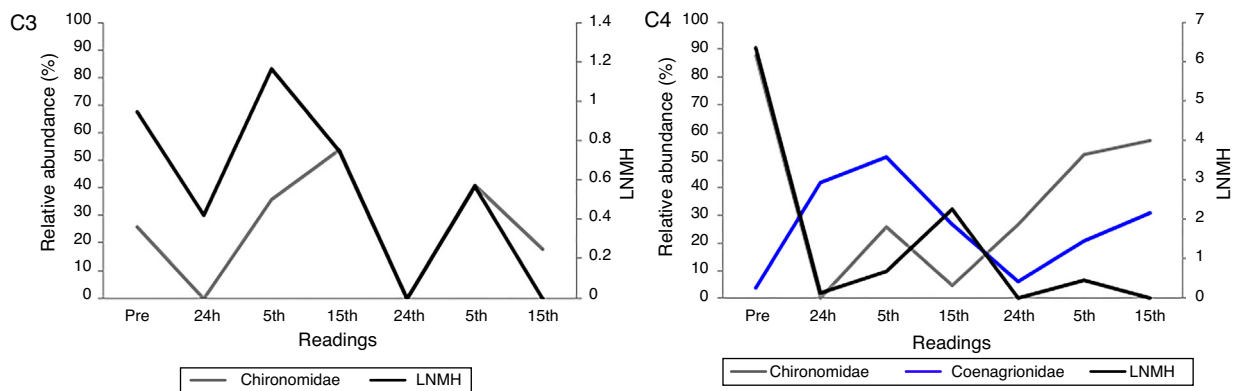


Fig. 3. Abundance over time of aquatic insects and values LNMH at C3 and C4, Manaus, Amazonas, Brazil.

The larval reduction data demonstrated the effectiveness of *B. sphaericus*, which eliminated larval breeding in three of the five sites analyzed 24 h after application. However, recolonization was observed at 15 days after the application, indicating the short persistence of biolarvicide in these environments (Table 2).

#### Evaluation of associated insect fauna

As a parameter of the effects of *B. sphaericus* on breeding, larval density values of *Anopheles* sp. (LNMH) were superimposed. There was variation in the abundance of specimens of Chironomidae and Coenagrionidae at C1, but 24 h after application of biolarvicide, the families were found exceeding the pre-implementation collection number. At C2 24 h after the application of biolarvicide, the abundance of Chironomidae increased compared to the values found in pre-sampling (Fig. 2).

At C3, the population of chironomids changed in abundance, which accompanied the larval density of *Anopheles*. At C4, two families were dominant, Chironomidae and Ceratopogonidae, both

demonstrating variability in relative abundance during the experiment, but at the end, both showed high levels (Fig. 3).

At C5, chironomids also varied in their population throughout the experiment, but the abundance found before applying biolarvicide was less than that found at the end of the experiment, with no elimination of these individuals throughout the experiment (Fig. 4).

The model that best fit was a 2nd order polynomial regression, described by the following equations.  $y = a + b \cdot x$ , where  $a = 0.54$ ,  $b = 0.001$  ( $r^2 = 0.002$ ), for diversity,  $y = a + b \cdot x$ , where  $a = 12.21$ ,  $b = 0.21$  ( $r^2 = 0.047$ ), for richness (Fig. 5). The line does not indicate a negative variation of the variables analyzed, diversity followed a constant and the richness index showed a slight increase, possibly because of the large variation in the data between the points.

Table 2

Larval reduction (%) found in the breeding of *Anopheles* after application and reapplication of *Bacillus sphaericus*, Manaus, Amazonas, Brazil.

Fish pond	Application of readings			Reapplication of readings		
	24 h	5 days	15 days	24 h	5 days	15 days
C1	100	100	88	100	100	85
C2	100	98	23	98	98	78
C3	56	–23	22	100	40	100
C4	98	89	65	100	93	100
C5	100	81	69	100	97	100

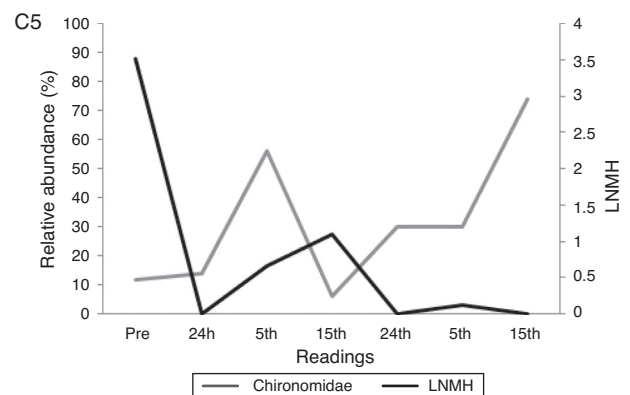


Fig. 4. Abundance over time of Chironomidae and values LNMH at C5, Manaus, Amazonas, Brazil.



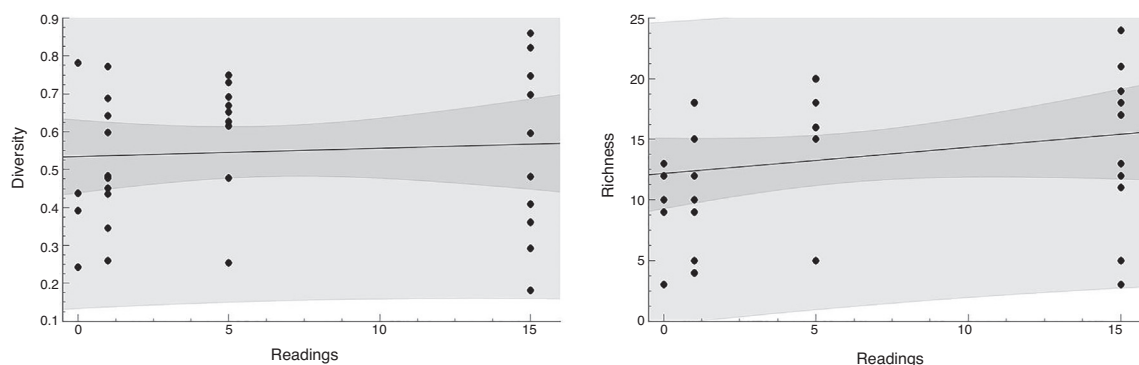


Fig. 5. Regression analysis of diversity and richness data compared to application of *Bacillus sphaericus*.

This data shows that there has been no effect of *B. sphaericus* on the insect fauna associated with this study.

## Discussion

The proximity of the urban area is possibly the biggest factor that determines the abundance of *A. darlingi*, since this species has high anthropophily (Tadei et al., 1998). The order Diptera was the most abundant at four of the five breeding sites analyzed; it is commonly dominant in both lotic and lentic environments due to its tolerance to extreme conditions such as hypoxia and also because of its strong competitive ability (Nessimian, 1995; Callisto et al., 2001).

According to Amorim and Castillo (2009), chironomids show generalist and opportunistic feeding habits, mainly being collectors-gatherers, which most often utilize periphyton organisms as food, a fact that explains the dominance of this group over the other taxa, at most breeding sites.

A large number of collectors are associated with the presence of wetland and riparian vegetation at breeding sites, and according to Koetsier and McArthur (2000), macrophytes play an important role in the retention of organic matter, favoring the presence of this trophic group. Data from the present study corroborate those of Merritt et al. (2005) in studies on the application of *B. sphaericus* on the associated insect fauna, who found that of the total aquatic insects sampled, the majority belonged to the collector group.

The low frequency of scrapers and shredders in the environments studied groups was influenced by the high abundance of Chironomidae, which according to the literature, compete for food (fine particulate organic matter) and space for shelter and protection (Amorim and Castillo, 2009).

Wilson (1991) and Peterson (1992) stated that in natural environments, the greater the presence of predators, the less the competition is between organisms, resulting in increased diversity. However, in the fish ponds studied, the greater abundance of predators did not increase the diversity, possibly because it is an isolated environment with a limited number of niches to be occupied.

The LNMH values found prior to samplings in this study were low compared to those found by Rodrigues et al. (2008) in *B. sphaericus* applications in fish ponds (mean 13.7). However, our findings corroborate those regarding the decrease in density of larvae 24 h after larvicide application.

Considering the results of larval reduction at breeding site C3, no high mortality was observed in the first days post-application. The marginal vegetation influenced the larvicidal activity since all the breeding site margin was covered by *Brachiaria* sp., thus increasing the amount of organic matter. This finding corroborates the results obtained by Alves et al. (2006) who reported low activity for VECTOLEX® larvicide at organically enriched breeding sites of *Culex* sp.

The variables studied here reinforce the specificity of *B. sphaericus* for *Anopheles* and *Culex*, as observed by Mulla et al. (1984), Aly and Mulla (1987), Lacey and Mulla (1990), Becker (1997), Lacey and Siegel (2000) and Lacey and Merritt (2003), who also found no effect of *B. sphaericus* on chironomids, other Diptera families and predators of mosquitoes in the field. The family Chironomidae was possibly not affected due to the benthic habitat as larvae, causing it to have no contact with the VECTOLEX that when applied on the surface of the water is due to its composition of corn cobs.

Finally, the fish pond breeding habitat was also associated with insect fauna that was not affected by the application of *B. sphaericus* over 30 days. However, studies monitoring water bodies for a longer period, with repeated applications and other formulations of biolarvicide, should be conducted to observe the behavior of aquatic populations.

## Conflicts of interest

The authors declare no conflicts of interest.

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